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Article (Accepted Version)

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E.L. Baple[1], H. Chambers[2], H. Cross[3], H. Fawcett[4], Y. Nakazawa[5,6], B.A. Chioza[1], G.V. Harlalka[1], S. Mansour[7], A. Sreekantan-Nair[1], M.A. Patton[1], M. Muggenthaler[1], P. Rich[8], K. Wagner[9], R. Coblentz[9], C.K. Stein[10], J.I. Last[11], A.M.R. Taylor[11], A.P. Jackson[12], T. Ogi[5,6], A.R. Lehmann[4], C.M. Green[2,13*], A.H. Crosby[1*]

Affiliations:

[1] Medical Research, RILD Wellcome Wolfson Centre, University of Exeter Medical School, Barrack Road, , EX2 5DW, Exeter, Devon, UK., Exeter, Devon, UK.

[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK.

[3]Department of Ophthalmology, University of Arizona College of Medicine, 655 North Alvernon Way, Tucson, AZ 85711, USA.

[4]Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton, BN1 9RQ, UK.

[5]Nagasaki University Research Centre for Genomic Instability and Carcinogenesis (NRGIC), Nagasaki, Japan.

[6]Department of Molecular Medicine, Atomic Bomb Disease Institute, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan.

[7]SW Thames Regional Genetics Service, St George's Healthcare NHS Trust, London SW17 0QT, UK.

[8]Department of Neuroradiology, St George's Hospital, London SW17 0QT, UK.

[9]Windows of Hope Genetic Study, Holmes County, OH 44687, USA.

[10]SUNY Upstate Medical University, 3813 Upstate University Hospital, 750 East Adams Street, Syracruse, NY 13210, USA.

[11]School of Cancer Sciences, College of Medical and Dental Sciences, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TT, UK.

[12]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK.

[13]Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, UK

*Correspondence should be addressed to:
Prof. Andrew H Crosby
Professor of Human Genetics
Medical Research (Level 4),
RILD Wellcome Wolfson Centre,
Royal Devon & Exeter NHS Foundation Trust,
Barrack Road, Exeter, EX2 5DW, UK

TEL: +44 (0)1392 408302
FAX: +44 (0)1392 408388
Email: A.H.Crosby@exeter.ac.uk

and

Dr. Catherine Green
Head of Core in Chromosome Dynamics
Wellcome Trust Centre for Human Genetics
University of Oxford
Roosevelt drive, Oxford, OX3 7BN
Tel: 01865 287510
Fax: 01865 287501
Email: catherine.green@well.ox.ac.uk

Conflict of Interest: The authors have declared that no conflict of interest exists.

ABSTRACT

A number of human disorders, including Cockayne syndrome, UV-sensitive syndrome, xeroderma pigmentosum and trichothiodystrophy, result from the mutation of genes encoding molecules important for nucleotide excision repair. Here, we describe a novel syndrome in which the cardinal clinical features include postnatal growth retardation, hearing loss, premature aging, telangiectasia, neurological signs and photosensitivity, resulting from a homozygous missense (p.Ser228Ile) sequence alteration of the proliferating cell nuclear antigen (PCNA). PCNA is a highly conserved sliding clamp protein essential for DNA replication and repair. Due to this fundamental role, mutations in PCNA that profoundly impair protein function would be incompatible with life. Interestingly, while the p.Ser228Ile alteration appears to have no effect on protein levels or DNA replication, patient cells exhibit significant abnormalities in response to UV irradiation displaying substantial reductions in both UV survival and RNA synthesis recovery. The p.Ser228Ile change also profoundly alters PCNA's interaction with Flap endonuclease 1 and DNA Ligase 1, DNA metabolism enzymes. Taken together our findings detail the first mutation of PCNA in humans, associated with a unique neurodegenerative disease displaying clinical and molecular features common to other DNA repair disorders, which we show to be attributable to a hypomorphic amino acid alteration.

INTRODUCTION

Maintenance of genomic integrity is fundamentally important for normal cell division and basic cellular biological processes. The molecular characterization of human DNA damage sensitivity disorders such as ataxia telangiectasia (AT), xeroderma pigmentosum (XP) and Cockayne syndrome (CS) has provided unique insight into the cellular mechanisms required for DNA damage tolerance and repair, and their importance for human health. The key features of such syndromes include predisposition to premature aging, malignancy, neurodegeneration, immunodeficiency, photosensitivity and growth insufficiency (1-4). The process of nucleotide excision repair (NER) comprises two DNA repair pathways that are crucial for the removal of bulky lesions in DNA including photoproducts resulting from exposure to ultraviolet (UV) light. Transcription-coupled nucleotide excision repair (TC-NER) rapidly undertakes preferential repair of DNA lesions on actively transcribed DNA strands and is deficient in CS and UV-sensitive syndrome (5). Global genome repair (GG-NER), removes photoproducts more slowly from across the genome and is deficient in XP and trichothiodystrophy (6). After DNA damage is detected, both NER processes involve the excision of about 30 nucleotides encompassing the damaged DNA followed by repair synthesis of new DNA to replace the damaged section. Repair synthesis is absolutely dependent on the DNA polymerase accessory protein PCNA (7). This protein's major cellular role is to recruit and retain the replicative DNA polymerases at the sites of DNA synthesis during DNA replication. It forms a homotrimeric ring encircling and freely sliding along the DNA helix. PCNA interacts with a large number of accessory proteins

and acts as a protein recruitment platform to coordinate the multiple enzymatic activities that are required for DNA replication and repair (8).

In the current study, four affected individuals aged between 11 and 31 years affected by a novel syndrome, were identified from a single extended pedigree comprising two kindreds within the Ohio Amish community (Figure 1). The principal features include neurodegeneration, postnatal growth retardation, prelingual sensorineural hearing loss, premature aging, ocular and cutaneous telangiectasia, learning difficulties, photophobia and photosensitivity with evidence of a predisposition to sun-induced malignancy. Phenotypic similarities with XP, CS and AT were noted (Table 1). All four affected individuals displayed short stature ranging from -3.8 to -5.2 SDS, with an absence of pubertal growth spurt in the two eldest individuals. Neurodegeneration was a consistent feature, characterized by progressive gait instability, muscle weakness, foot deformity, difficulties with speech and swallowing, learning difficulties and cognitive decline with advancing age. Prelingual onset of moderate to profound (worse at high frequency) sensorineural hearing loss was universal, a feature not commonly associated with the recognized DNA repair defects, and there was clear evidence of progression in VI:9. Muscle weakness was progressive (VI:6 has been wheelchair bound since the age of 16 years with contractures in all 4 limbs). Speech was unclear from onset and deteriorated with age (VI:6 currently has no speech and communicates with basic gestures). Progressive difficulty with swallowing solids/drooling was present in 3/4 affected subjects. Neuroimaging was only available in one case (VI:7). Magnetic resonance imaging of the brain aged eight years demonstrated cerebellar atrophy involving the cerebellar vermis and hemispheres. Inner ear abnormalities included

bilateral mild labyrinthine dysplasia with mildly dilated vestibules. All exhibited photophobia and photosensitivity, with evidence of premalignant changes in VI:11 (basal cell carcinoma *in situ*). All have several cutaneous telangiectasias and 3/4 have conjunctival telangiectasia.

Assuming that a founder mutation was responsible for the condition, we used a combination of autozygosity mapping and linkage analysis in order to study this novel syndrome and identify the underlying molecular cause.

RESULTS

A genome-wide SNP microarray scan of DNA from affected individuals and parents was undertaken. Inspection of resultant genotypes identified a single notable region of homozygosity of ~2.72Mb on chromosome 20p13, shared solely by the three affected females. In the affected male, a *de novo* telomeric recombination event reduced this region to ~0.77Mb, delimited by markers rs404678 and rs6139841, NC_000020.10:g.5024237-5792923 (Figure 1), likely to correspond to the disease locus (LOD_{MAX} 6.4). Microsatellite marker analysis confirmed autozygosity across this region (data not shown). We sequenced all six protein coding genes in the region (*PCNA, CDS2*, *PROKR2, GPCPD1*, *C20orf30 and C20orf196*), revealing only a single potentially pathogenic sequence variant (NM_002592.2 c.683G>T) in exon 6 of *PCNA.* This variant, which cosegregated with the disease phenotype and was verified in the RNA transcript obtained from whole blood of an affected individual, is predicted to result in a substitution of a stringently conserved serine at position 228 for isoleucine

(p.Ser228Ile) (Supplemental Figure S1). The variant was not detected in 360 control chromosomes of European ancestry and only two heterozygous carriers were detected in the analysis of 310 Ohio Amish control chromosomes, which is not unexpected in this endogamous community. Only a single heterozygous carrier of European origin was identified in online sequencing project databases (1000 genomes project and the Exome Variant Server, ESP6500SI release). This single mutation carrier in 7775 non-Amish individuals genotyped corresponds to a homozygous *PCNA* c.683T allele incidence in the region of ~1:241 million. We also subjected a single affected individual to whole exome sequence analysis (Otogenetics Corporation) to exclude other possible genetic causes. As expected this confirmed the presence of the PCNA variant but identified no other known or possible pathogenic variants elsewhere after excluding variants not compatible with recessive inheritance or cosegregating with the disease phenotype. Further conclusive support for the exclusion of a diagnosis of AT and "AT-like" disorders was provided by our findings including normal chromosomes (untreated or γ-ray-induced, patient VI:11), AFP levels, immunoglobulin patterns (VI:7 and 9), ATM and associated protein and kinase activity levels (patients VI:6 and 11) (Supplemental Figures S2, S3) in affected subjects.

PCNA is an essential DNA replication accessory protein, highly conserved throughout evolution. It plays a central role at the replication fork, recruiting and retaining many of the enzymes required for DNA replication (8). Due to its fundamentally important cellular role, mutations that significantly affect PCNA protein function would be predicted to result in embryonic lethality. However the survival of $PCNA^{Lys164Arg/Lys164Arg}$ knock-in mice, generated to determine the role of PCNA lysine 164 modifications in somatic

hypermutation, demonstrates that certain hypomorphic mutations of PCNA can be tolerated in mammals (9). However, no inherited human disorder arising from PCNA mutation has been described previously.

We therefore investigated the functional consequences of the p.Ser228Ile PCNA alteration. Primary fibroblasts were established from three affected individuals, VI:11, VI:9 and VI:7 (P1, P2, P3), two heterozygous carriers (Het1, Het2) and wild type controls (WT). EBV transformed lymphoblastoid cells were established from two affected individuals VI:11 and VI:6 (L5, L6), two carriers (L8, L10) and two Amish wild type controls (L7, L9). PCNA protein levels are unaltered in cells from affected individuals (Supplemental Figure S2). In order to assess the influence of the p.Ser228Ile alteration on DNA replication we incubated primary fibroblasts (at < passage 10) with BrdU (5-Bromo-2´-Deoxyuridine) for 30 minutes and analyzed the incorporation and DNA content by FACS (Figure 2A). No significant differences were observed when comparing the fibroblasts from affected individuals with wild type or heterozygote controls. During S phase, PCNA is recruited to so called “DNA replication factories”, which are the sites of nucleotide incorporation and are visible as foci in the nuclei of replicating cells. Immunofluorescence analysis of endogenous PCNA in fibroblasts from affected individuals revealed no abnormalities in these foci (Figure 2B). In addition, no difference in replication fork rate was detected during unperturbed S phase using DNA fiber analysis (Figure 2C). Taken together, these data lead us to conclude that the Ser228Ile variant does not dramatically interfere with the major replicative function of PCNA and that bulk DNA replication is not grossly perturbed.

Given that repair synthesis and downstream events of NER are absolutely dependent on PCNA (7), the reported photosensitivity in p.Ser228Ile homozygotes prompted us to examine the effect of the p.Ser228Ile substitution on cellular sensitivity to UV. Both fibroblast and lymphoblastoid cells from affected individuals are more sensitive to UV irradiation than cells with wild-type PCNA (Figure 3A, 3B), suggesting that the mutation specifically impairs the function of PCNA in DNA repair. To investigate this further, we evaluated GG-NER in primary fibroblast cell lines, measured by the unscheduled DNA synthesis (UDS) assay (10). In cells from affected individuals UDS was reproducibly reduced to approximately 50-60% of normal values (Figure 3C). RNA synthesis recovery levels following UV radiation (RRS) were used as a marker of TC-NER (11). RRS was significantly decreased, to approximately 30-50% of normal in cells from affected individuals (Figure 3D, Supplemental Figure S4). The deficiency approaches that of CS cells, known to be defective in TC-NER. Importantly, ectopic expression of wild-type PCNA fully rescued the RRS deficiency (Figure 3E) (12), whereas it had only a minor effect on RRS in normal fibroblasts (Supplemental Figure S5), demonstrating that the deficient UV responses are caused by the p.Ser228Ile sequence alteration. Although the two assays for UDS and RRS are, by their very nature, different from each other and are semi-quantitative, these results suggest that a subtle alteration in PCNA structure resulting from the p.Ser228Ile substitution is sufficient to specifically disrupt its role in NER and this appears to have a more significant effect on TC-NER.

Serine 228 lies near the external face of PCNA and the mutation is therefore unlikely to affect its interaction with DNA (Figure 4A). This residue is also distant from the regions of PCNA that mediate the formation of the trimeric ring structure (13). Consistent with

this, no differences in trimer formation or stability between recombinant $PCNA^{WT}$ and $PCNA^{Ser228Ile}$ proteins were detected using gel filtration or glycerol gradient sedimentation (data not shown), and the amount of chromatin-associated PCNA (resistant to detergent extraction) was similar in wild-type and p.Ser228Ile homozygous cells. Interestingly however, the altered residue is close to the inter-domain connecting loop (IDCL) region mediating the interaction of PCNA with many of its protein partners. PCNA partner proteins, including DNA polymerase delta (PolD), DNA Ligase 1 (Lig1) and Flap endonuclease 1 (Fen1), often contain a PCNA interacting protein (PIP) box, a sequence motif which mediates the IDCL-interaction (14). In published crystal structures serine 228 does not directly contact the PIP box-containing peptide (13, 15), although its close proximity to the interaction region may generate subtle positional alterations within this crucial domain, with functional consequences.

To determine whether specific protein interactions are perturbed by the p.Ser228Ile alteration, we performed a SILAC (stable isotope labeling by amino acids in culture) affinity purification assay. Extracts were made from HeLa cells grown in either normal medium, or SILAC medium containing "heavy" amino acids: [$^{13}C_6/^{15}N_2$] lysine and [$^{13}C_6/^{15}N_4$] arginine. These extracts were individually exposed to affinity columns comprising recombinant PCNA, either wild-type (WT) or $PCNA^{Ser228Ile}$. Proteins that bound to each column were eluted, the eluates combined, and the relative binding to each column was determined by mass spectrometry. This identified clear perturbations in the interaction of $PCNA^{Ser228Ile}$ with a limited number of interacting partners, most strikingly with Fen1 and Lig1 (Figure 4B, Supplemental Table S1). This experiment revealed relatively increased amounts of Fen1 and Lig1 in the 500mM eluates from the

PCNA^{Ser228Ile} column, when compared to the WT column. To investigate this unexpected finding further, we analyzed the salt resistance of the PCNA-Fen1 interaction. HeLa nuclear extract was incubated with His-PCNA (WT or p.Ser228Ile) beads, which were then washed with increasing NaCl up to 500mM. The material eluted at 1M NaCl and that which was retained on the beads was subsequently analyzed by western blot for Fen1 (Figure 4C). This clearly demonstrates that the interaction between Fen1 and WT PCNA is much more resistant to high salt concentrations than the interaction between Fen1 and PCNA$^{p.Ser228Ile}$. This leads us to conclude that in the SILAC experiments (Figure 4B), Fen1 remained bound to the WT-PCNA column during the elution with 500mM NaCl, resulting in a dramatic reduction of this protein in the WT eluate. This conclusion, that the p.Ser228Ile change impairs the ability of PCNA to bind Fen1, was further validated by our subsequent immunoprecipitation and GST pull down experiments (described below).

We next assessed the effects of the p.Ser228Ile PCNA alteration in a more physiological setting by immunoprecipitation from lymphoblastoid cell extracts. While an interaction between Fen1 and PCNA was clearly demonstrated in controls, an intermediate level of interaction was detected in heterozygote cell lines and the interaction was barely detectable in cells homozygous for the Ser228Ile PCNA alteration (Figure 4D). These data provide unambiguous confirmation that interaction of Fen1 with PCNA is dramatically reduced by the p.Ser228Ile mutation.

We have been unable to identify a commercially available antibody against Lig1 that is effective for immunoprecipitation from our lymphoblastoid extracts. Thus, in order to further investigate the effect of the Ser228Ile PCNA alteration on the interaction

between PCNA and Lig1 we utilized an *in vitro* approach (16). Lysates were prepared from *E.coli* expressing a fusion protein comprising GST and the PIP box motif of either Fen1 or Lig1. These were then mixed with lysate containing either wild type or p.Ser228Ile PCNA and glutathione beads were added, the proteins that associated with the beads were subsequently analyzed by SDS-PAGE. This assay recapitulates the defective interaction between $PCNA^{Ser228Ile}$ and Fen1 (Figure 4E), and most significantly shows that the Ser228Ile protein also has a reduced affinity for the PIP box of Lig1 (Figure 4F, left panel).

Given that Fen1 and Lig1 are both implicated in the NER pathways (17, 18), it is plausible that the cellular NER defects observed in cells from affected individuals result from the compromised interactions of these proteins with p.Ser288Ile PCNA. It also seemed likely that the Ser228Ile alteration would perturb the PCNA interaction with other PIP box containing partners, and that this may contribute to the cellular phenotypes observed. One clear candidate known both to be required for NER and to bind PCNA through a PIP box interaction is XPG (16). We did not detect peptides derived from XPG in our SILAC assay, which by its nature will only detect proteins which are relatively abundant and interactions which are relatively strong. In the absence of an XPG antibody suitable for immunoprecipitation of endogenous XPG, the GST assay was again used to assess the binding of WT PCNA and p.Ser228Ile to the PIP box of XPG. Crucially, although less WT PCNA was bound to the XPG PIP box when compared to its binding to the Fen1 PIP box, the pSer228Ile PCNA displayed no detectable interaction with the XPG PIP box (Figure 4F, right panel). Thus, it seems

reasonable to suggest that a defective interaction with XPG is also likely to contribute to the NER phenotype seen in cells homozygous for p.Ser228Ile PCNA.

DISCUSSION

The genetic and cellular data described here implicate PCNA in a new distinct neurodegenerative DNA repair disorder displaying some features in common with other conditions associated with deficient DNA metabolism including CS, XP and AT. The most notable similarities between CS, the neurological form of XP and the individuals with PCNA mutation described here include growth abnormalities, premature ageing, cognitive decline, photosensitivity and photophobia. The presence of a basal cell carcinoma *in situ* in one affected individual (VI:11) likely indicates a predisposition to sun-induced malignancy in individuals homozygous for PCNA p.Ser228Ile mutation, and further clinical overlap with XP. However the phenotype associated with p.Ser228Ile mutation of PCNA appears in general to be milder than that seen with either typical CS or XP, most noticeably the rate of premature ageing seems to be slower, with none of the ophthalmic manifestations associated with CS reported and the effects of UV sensitivity less significant. Interestingly, although hearing loss is found in CS and some XP patients, the age of onset is typically later (1, 4). Thus, prelingual sensorineural hearing loss may, in association with the other clinical features described, be a helpful diagnostic feature in patients with this condition. The ocular and cutaneous telangiectasia seen in patients with the PCNA p.Ser228Ile mutation are reminiscent of those seen in AT, as is the pattern and progression of neurological abnormalities,

although as with CS and XP, PCNA p.Ser228Ile homozygotes generally appear to be less severely affected than typical AT patients. As with CS and XP, there are also striking differences between the phenotype associated with PCNA p.Ser228Ile and AT, including an absence of both the immunodeficiency and oculomotor apraxia commonly seen in AT affected individuals.

Complete disruption of PCNA in flies or yeast is lethal (19, 20) an expected finding given its fundamental involvement in DNA replication. Until now, no pathological mutations in the PCNA gene have been reported in human populations. In other species however, some mutations of PCNA are compatible with life. For example, point mutations have been produced in the *S. cerevisiae* PCNA gene (*POL30*) that result in a wide variety of phenotypes, including DNA damage sensitivity, alterations in mutation rate and epigenetic silencing defects (21). Similar phenotypes are seen in the various reported mutations in Drosophila PCNA (*mus209*) (19). The only previously reported pathological mutation of PCNA in mammalian systems is a targeted missense mutation of lysine 164 to arginine ($PCNA^{p.Lys164Arg}$) in mice, generated to assess the role of ubiquitination at this residue in somatic hypermutation in the immune system (9). Ubiquitination of PCNA at lysine 164 is essential for translesion synthesis, a DNA damage tolerance process (22). Mice homozygous for $PCNA^{p.Lys164Arg}$ are viable, but infertile and have an altered mutation spectrum of hypermutated immunoglobulin genes (9, 23). Thus a subtle mutation of PCNA that impinges upon a specific function can lead to characteristic loss of function effects on DNA metabolism.

Consistent with this, our assays suggest that the PCNA p.Ser228Ile mutation described here is hypomorphic in nature. We did not detect any gross abnormalities of DNA replication associated with this mutation. Instead, we showed that the mutation has distinctive consequences for the cellular responses to UV, affecting both GG-NER and TC-NER. The effects on RRS seen in CS cell lines, and on UDS in XP cell lines, are typically more dramatic than we observed in homozygous PCNA p.Ser228Ile cells. This may in part explain why the phenotypic manifestations associated with these cellular abnormalities are not as profound in the individuals described here when compared to those seen in CS and XP.

Our studies define particular defective interactions of p.Ser228Ile PCNA which, together with others not yet recognized, are likely to be responsible for the specific alterations in cellular activities we observed. The altered protein interaction capability of the pSer228Ile PCNA is also likely to account for the clinical manifestations of this syndrome. Complete abrogation of the PCNA-Fen1 interaction, mediated by a homozygous mutation of the Fen1 PIP box, is lethal in mice (24). Hence, it seems likely that while the p.Ser228Ile alteration dramatically reduces the association between PCNA and Fen1 in cell extracts, it is unlikely to completely abrogate their functional partnership. Consistent with this, both our SILAC and interaction assays using recombinant proteins show that the p.Ser228Ile protein remains capable of interacting with Fen1, although with significantly altered affinity. We cannot conclude from our assays whether the defective interactions with XPG, Fen1 and Lig1 are solely responsible for the observed effects on NER, although it is important to note that all these proteins are reported to be involved in this repair pathway (6, 17, 18). Thus, the

perturbation of their interactions with PCNA could well be a major contributor to this abnormal cellular phenotype.

Mutations in XPG are rare, and can be divided into two categories: mis-sense mutations in the nuclease domains abrogate NER but result in relatively mild XP phenotypes, while truncating mutations cause a very severe XP/CS complex disorder (25, 26). The *xpg*$^{-/-}$ mouse is barely viable (27). These varying phenotypes are thought to result because, in addition to its function in NER, XPG is involved in other processes (26). In particular, it associates with the transcription factor TFIIH, and RNA polymerase II, and has a role in transcription (25, 28, 29). It is the disruption of these other functions of XPG that is thought to be responsible for the more severe features of truncation mutations. As an example, patient XPCS1RO had severe early-onset CS and died aged 7 months. He was homozygous for a frame-shift mutation at codon 926 of *XPG*. This mutation leaves the nuclease domains intact, but truncates the protein before the PIP-box and nuclear localization signal (30). Thus, the C-terminal region of XPG, including the motif responsible for interacting with PCNA, is very important for XPG function. These observations lead us to suggest that perturbation of PCNA's interaction with XPG may contribute to the neurological features associated with PCNA p.Ser228Ile.

In contrast to the homozygous state, mice heterozygous for the Fen1 PIP box mutant are viable, though they show predisposition to malignancy (31). Although specific PIP box mutations of Lig1 have not been generated in mammals, loss of function mutations in *LIG1* have been reported in a single individual and result in a complex human phenotype comprising growth restriction, immunodeficiency and photosensitivity (32), features reminiscent of those seen in p.Ser228Ile homozygotes. It is possible that Lig1

deficiency and PCNA p.Ser228Ile homozygosity together represent a group of conditions that are the result of disruption to multiple different DNA repair pathways. In addition, while the observed cellular defects correlate with the CS and XP like features of the syndrome, the clinical overlap with AT will require further investigation.

As well as being crucial to the maintenance of genomic integrity and prevention of neoplastic changes, DNA repair is known to be fundamentally important both during the rapid proliferative phase characteristic of early neurogenesis, and in the prevention of early cell death. The affected individuals with PCNA mutation described in this study display signs of neurodegeneration, a recognized feature of CS, AT as well as neurological forms of XP, although the pathological patterns seen in each are distinct. More detailed investigation of the altered biological processes resulting from the p.Ser228Ile PCNA alteration will thus provide invaluable insight into the biological basis of this novel human disorder, as well as the neurodegenerative disease mechanism(s) involved in DNA damage tolerance and repair disorders. Further, while mutations resulting in complete loss of function of PCNA in humans may be incompatible with life, it remains an intriguing possibility that additional sequence variants in this gene, affecting other distinct aspects of PCNA function, might be viable and result in a phenotype more or indeed less severe than that associated with the p.Ser228Ile alteration described here.

METHODS

Genetic studies. SNP genotyping was carried out using Illumina Human CytoSNP-12v2.1 330K arrays. Multipoint linkage analysis was performed with MERLIN (33) under a model of autosomal recessive inheritance with full penetrance, assuming a disease allele frequency of 0.0001. Unique primers for sequencing and microsatellite analysis (Sigma-Aldrich) were designed using online software Primer3web (34) using sequences from the UCSC Genome Browser. RT-PCR was carried out using Clontech™ One Step RT-PCR Kit following manufacturer's instructions, and bidirectional dideoxy DNA sequencing performed on an ABI3130 XLA capillary sequencer (Applied Biosystems) with analysis using Finch TV 1.4.0 (Geospiza Inc.) and Gene Tool 1.0.0.1 (Bio Tools Inc). Whole exome sequencing was performed by the Otogenetics Corporation using the SureSelect Human All Exon V4 (Agilent Technologies) exome enrichment kit on an Illumina HiSeq2000. The exome sequencing produced 31,783,299 mapped reads, corresponding to 93% of targeted sequences covered sufficiently for variant calling (>10X coverage, mean depth 45X).

Cell studies. Cells were cultured in DMEM/RPMI (Sigma Aldrich) with 15% fetal bovine serum (FCS - hyclone), 2mM Glutamine (Invitrogen) and 1% penicillin and streptomycin. UV sensitivity of lymphoblastoid cells was determined by irradiating cells in PBS ($1x10^6$ viable cells in 1ml) with UVC (254nm, $1Jm^{-2}sec^{-1}$), adding 4ml growth medium and counting viable cells using trypan blue exclusion after a 72 hour recovery period. UDS, RNA synthesis, fibroblast survival assays and lentiviral mediated complementation were performed as described previously (10-12, 35-37).

Protein interaction analyses. Recombinant PCNA (WT and p.Ser228Ile) were produced with a His-tag from pET28b in *E.coli* BL21 codonplus (Novagen), and purified on Ni-NTA sepharose (Qiagen). Affinity columns were made by covalently linking these proteins to NHS-activated sepharose (Pierce). For SILAC analysis HeLa cells were grown in SILAC DMEM (Gibco) supplemented with 10% dialyzed FBS, 2mM $CaCl_2$, 1mM $MgSO_4$, 52mg/l L-leucine and 100mg/l L-lysine and L-arginine (for "heavy" extracts [$^{13}C_6/^{15}N_2$] lysine and [$^{13}C_6/^{15}N_4$] arginine (CKgas) were substituted). Extracts were made by lysing cells in a low salt buffer containing benzonase (Merck), then adjusting to 150mM NaCl. All protein interaction assays were performed in interaction buffer (25mM Tris pH 7.5, 25mm NaCl, 10% glycerol, 0.01% Igepal, 1mM PMSF). Proteins eluted from PCNA affinity columns with 500 mM NaCl were combined before 10-fraction mass spectrometric (MS-MS) analysis (MS Bioworks). Data was analyzed, and H:L ratios determined using MaxQuant software. To further analyze the stability of PCNA interactions, recombinant PCNAs were added to HeLa nuclear extract (4C), complexes were isolated on Ni-NTA agarose, and sequentially washed and eluted with increasing [NaCl]. For immunoprecipitations, protein extracts were made from lymphoblastoid cells as above. Fen1 protein was precipitated using an anti-Fen1 rabbit antibody (EPR4459(2), GeneTex) and ProteinA/G dynabeads (Invitrogen). Proteins in the extracts and precipitates were analyzed by western blot with anti-PCNA (PC10, Abcam) or anti-Fen1 (4E7, GeneTex). GST fusion protein association studies were performed as in (16), using lysates from *E.coli* harboring pET30a as a control, or PCNA (WT or p.Ser228Ile) expressed from pET30a. These were mixed, in a buffer containing 100mM KH_2PO_4/K_2HPO_4, with lysates from *E. coli* expressing glutathione S transferase (GST)

or fusion proteins comprising GST and the PIP boxes of Fen1 (amino acids 328-355), XPG (amino acids 981-1009) or Lig1 (amino acids 1 – 21 with an 8 amino acid glycine-serine spacer to maintain an equal distance between the GST and the PIP box in all cases).

Western Blotting. Exponentially growing primary human fibroblast cells were lysed on ice in 50mM Tris-HCl pH 7.5, 150mM NaCl, 200μM EDTA, 1% NP-40. Extracts were clarified by centrifugation at 10,000g for 10 min at 4°C and extracts fractionated by SDS-PAGE. Proteins were transferred to Protran nitrocellulose (Whatman) and immunoblotted with anti-PCNA (PC10, Abcam) or anti-Fen1 (4E7, GeneTex). The ATM kinase activity assay and western blotting of ATM and associated proteins in lymphoblastoid cells was carried out as described in (38).

FACS analysis of cell cycle position. Primary fibroblasts were incubated with 10 μM BrdU for 30 minutes then trypsinized and fixed by the addition of methanol to 70%. After storage at -20 °C cells were treated with 0.2 mg/ml pepsin in 2M HCl for 20 minutes, then incubated in 0.5% BSA in PBS with 0.5% tween 20 and a 1/50 dilution of anti-BrdU antibody (Becton Dickinson) for 1 hour. After washing in 0.5% BSA, incubation with 1/100 anti-mouse secondary antibody coupled to Alexa Fluor 488 (Molecular Probes) was for 30 minutes, followed by washing and resuspension in PBS with 0.5 mg/ml RNaseA for 15 minutes. Propidium iodide was added (10 μg/ml) just before analysis on a CyAn™ ADP Analyzer (Beckman Coulter, Inc.).

Immunofluorescence. EdU staining was carried out using the Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen). Cells were seeded onto glass coverslips so as to be

subconfluent at the time of fixation. At least 24 h after plating EdU was added to the culture medium to a final concentration of 20 μM and cells returned to 37°C for 10 min. Soluble proteins were extracted by rinsing once in PBS, once in CSK buffer (100mM NaCl, 300mM sucrose, 10mM PIPES pH 7.0, 3mM MgCl2), and then soaking in CSK plus 0.2% Triton X-100 for 5 min on ice. After a further wash in CSK cells were fixed (ice-cold methanol for 20 min at -20°C), washed twice more in PBS, and then blocked (5% w/v bovine serum albumin (BSA) in PBST) for 30 min at room temperature. EdU staining was carried out according to manufacturer's instructions. Coverslips were then incubated for 1 h at room temperature in blocking buffer containing anti-PCNA monoclonal antibody (PC-10) diluted 1:1000, followed by 3 washes in PBST and incubation in Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Invitrogen) diluted 1:1000 in blocking buffer for 45 min. Cells were rinsed a further 3 times in PBST, and coverslips mounted in Aqua-Poly/Mount (Polysciences) containing DAPI at a final concentration of 1.5μg/ml. Slides were visualized using a Leica SP5 confocal microscope.

DNA fiber analysis. Immunolabelling of DNA fiber spreads was carried out as described in (39), with minor modifications. Cells were incubated in IdU and CldU at a final concentration of 100 μM for 20 min each, and all antibody incubations were for 2 h at room temperature. Fibers were mounted in ProLong mounting medium (Invitrogen).

Study approval. The present studies in humans were reviewed and approved by The Institutional Review Board of the Office for the Responsible Conduct of Research, University of Arizona, Tucson, Arizona (reference 10-0050-01).

All tissue samples were taken with informed consent in accordance with all ethical standards and protocols. Subjects or their guardians provided informed consent prior to their participation in the study. Written informed consent was obtained to publish the photographs of affected individuals.

Statistics overview. In graphical representations of experimental data, error bars on graphs represent the standard error of the mean (SEM) or range of the plotted mean of at least 2 independent experiments, as indicated in the figure legends.

ACKNOWLEDGMENTS

First and foremost we are grateful to the families for partaking in this study, and to the Amish community for their continuing support of the Windows of Hope project. We are grateful to Dr Luther Robinson for contributing clinical data, Dr Kristiana Gordon for review of the dermatology, Dr Wendy Albuquerque for review of the audiograms and Jon Wing for the data in Figure 3A. Chromosome breakage studies were carried out at GSTS Pathology, and karyotyping at South West Thames Genetics Laboratory. Illumina cytoSNP analysis and Sanger sequencing were carried out at the Medical Biomics Centre, St George's University of London. The FACS analysis was done by Drew Worth in the Flow Cytometry Core Facility of the Jenner Institute, Oxford. The work was supported by MRC Clinical Research Training fellowship (G1001931 to E.L.B), MRC grant (G1002279 to AHC), MRC centenary fund award (G0900205, St George's University of London, E.L.B), Newlife Foundation for disabled children (E.L.B and A.H.C), a Cancer Research UK career development fellowship (C24125/A8307 to CMG) and the Wellcome Trust (090532/Z/09/Z to CMG).

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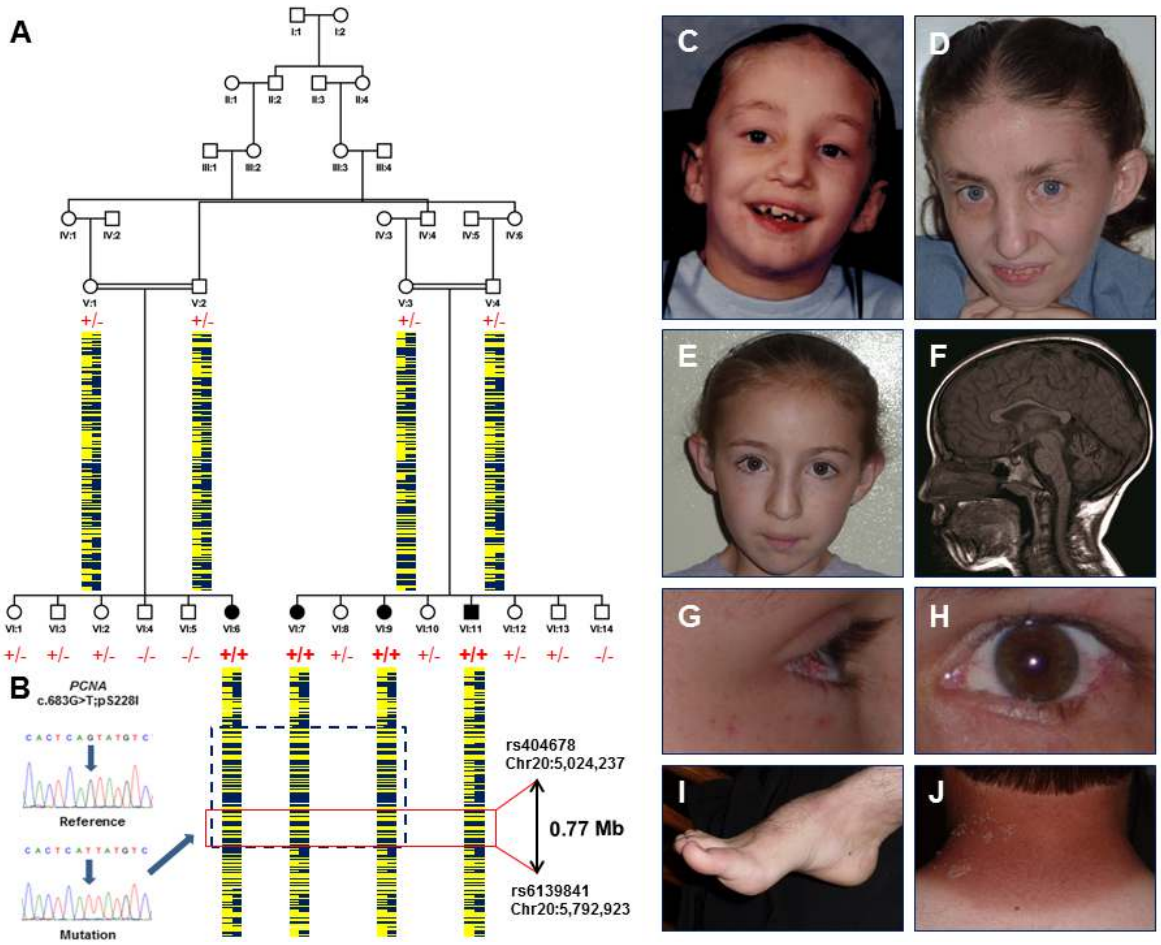

Figure 1: Family pedigree showing PCNA c.683G>T genotype data, and images of affected individuals. (A) A simplified pedigree of the extended Amish family investigated, with pictorial representation of genotypes across ~6Mb of chromosome 20 encompassing the disease locus (2.7Mb autozygous section in affected females indicated by blue hashed box, and the common 0.78Mb region indicated by red box). All affected individuals were subsequently shown to be homozygous for the PCNA (NM_002592.2 c.683G>T) variant (indicated). Parental samples were heterozygous and unaffected siblings were either wild type or heterozygous carriers. The genotype is shown in red under individuals in generations V and VI, with (+) denoting mutant and (-)

wild type. **(B)** Electropherograms showing the DNA sequence at the position of the (NM_002592.2 c.683G>T) PCNA variant in a wild-type control, and homozygous affected individual. **(C-J)** The clinical features of individuals homozygous for *PCNA* c.683G>T. Individual VI:6 aged 8 years **(C)** and 31 years **(D)** showing signs of premature aging. **(E)** Individual VI:7 aged 11 years with bilateral hearing aids insitu. **(F)** Midline sagittal T1-weighted brain scan (individual VI:9 aged 8 years). There is atrophy of the cerebellar vermis resulting in enlargement of vermian sulci and mild widening of the fourth ventricle. The brainstem is normal in appearance and no supratentorial abnormality is shown. **(G** and **H)** Ocular and cutaneous telangiectasia (Individuals VI:7 and VI:11). **(I)** Pes Cavus (Individual VI:11). **(J)** Photosensitivity after minimal sun exposure (individual VI:11).

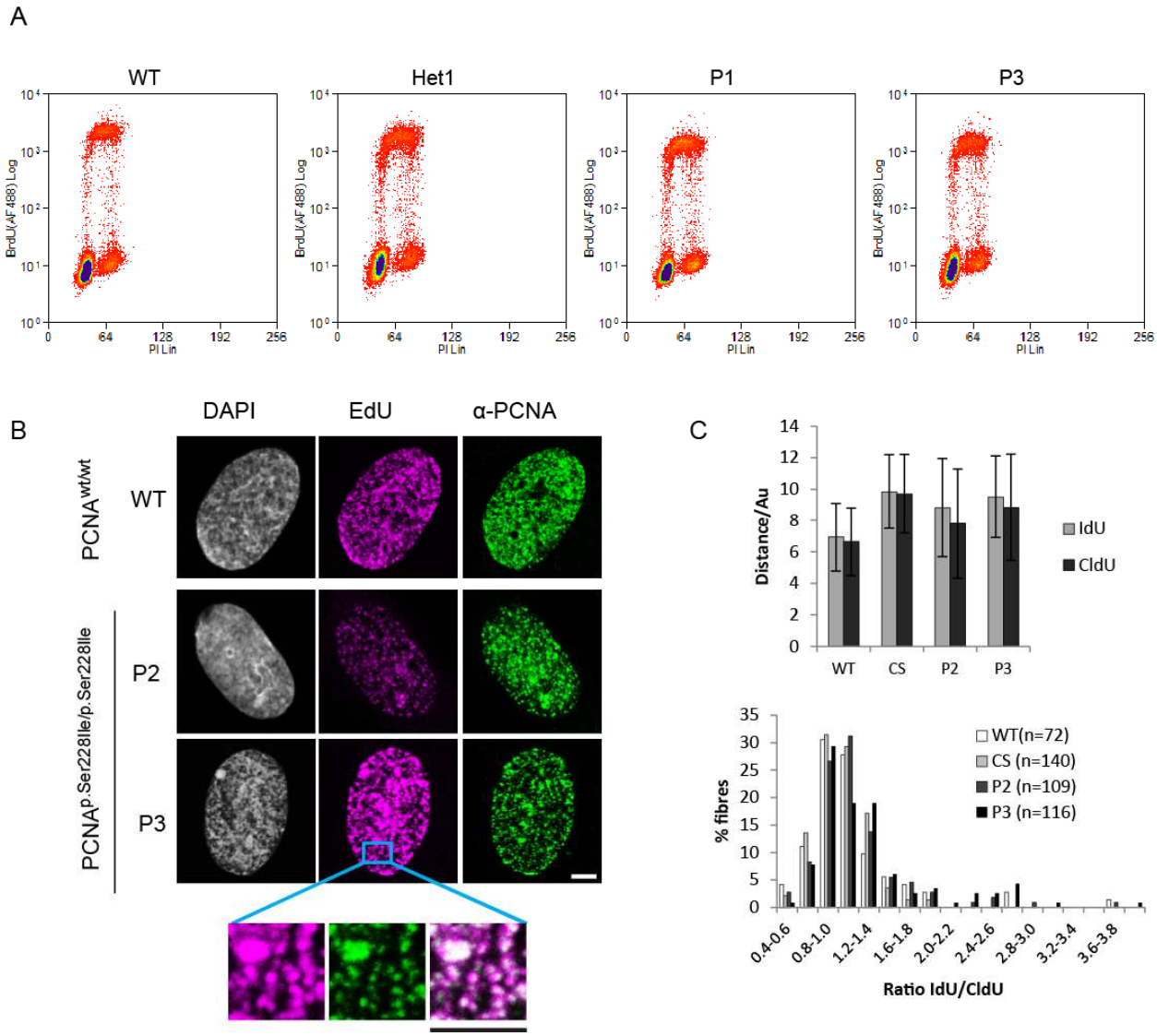

Figure 2: Cells homozygous for PCNA p.Ser228Ile have normal replication parameters. (A) FACS analysis of S phase in primary cells. Cells (passage number <10) were labelled with BrdU for 30 minutes and BrdU incorporation and DNA content analyzed by FACS. No significant differences were observed between cells from affected individuals (P1, P3) and related (Het1) or unrelated (WT) control cells. Representative scatter plots are shown, each line was independently analyzed at least

twice. **(B)** Immunofluorescence of primary control cells (WT), Cockayne syndrome cells (CS) and affected individuals homozygous for PCNA p.Ser228Ile (P2 and P3). Soluble proteins were removed by triton extraction prior to fixation, and cells were immunostained for PCNA (right hand panels). The center panel shows sites of active DNA replication, visible as foci of incorporation of EdU (5-ethynyl-2´-deoxyuridine). Enlargement shows colocalization between these signals. DNA was counterstained with DAPI (left hand panel). Scale bars = 3μm. **(C)** Analysis of replication fork progression. Primary control cells and Cockayne syndrome cells (WT and CS) and cells homozygous for PCNA p.Ser228Ile (P2 and P3) were incubated sequentially in medium supplemented with IdU (5-iodo-2'-deoxyuridine) and CldU (5-chloro-2'-deoxyuridine), and the length of the DNA fiber replicated during each pulse measured. Comparable lengths of DNA were replicated during each pulse (top panel, compare light grey [IdU] and dark grey [CldU] bars for each cell line). The frequency distribution of the ratio of IdU/CldU within each fiber was similar between cell lines (bottom panel). The error bars indicate SEM of two experiments.

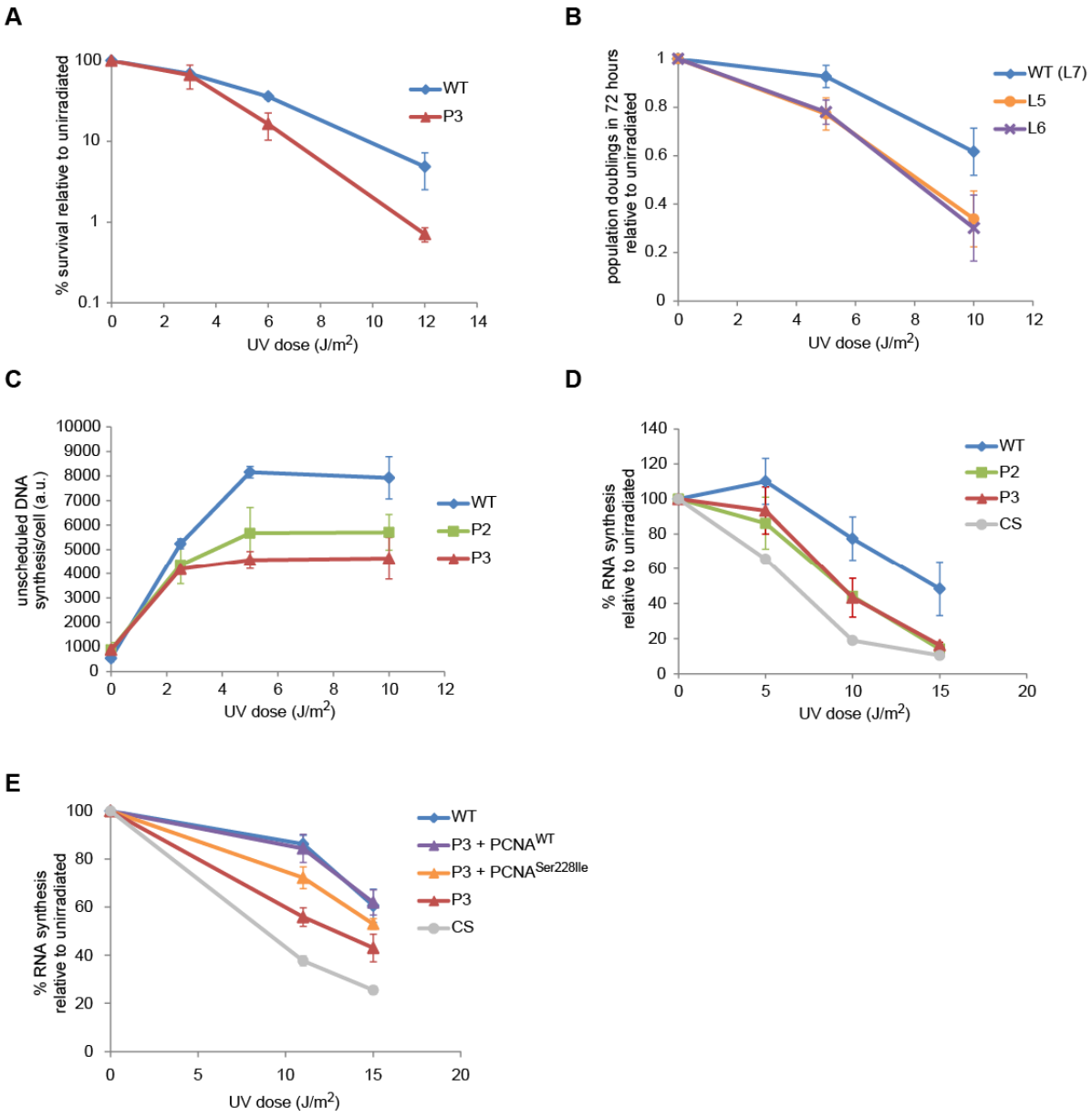

Figure 3: Abnormal cellular responses to UV. (A) Primary fibroblasts from an affected individual are sensitive to UV. Cell survival was measured by colony-forming ability following exposure to the indicated doses of UVC. Plotted is the mean and range of two experiments. **(B)** Lymphoblastoid cells from two different affected individuals are sensitive to UV. Viable cells were counted 72 hours after exposure to UVC using trypan blue exclusion. Mean of three experiments +/- SEM. **(C)** Cells from affected individuals

have reduced UDS activity. Unscheduled DNA synthesis measured by incorporation of radiolabelled thymidine immediately after UV-irradiation of non-dividing cells with the indicated doses of UVC. Means ± SEM of three experiments are shown. **(D)** RNA synthesis measured by the incorporation of radiolabelled uridine 24 h after UVC-irradiation of non-dividing cells with indicated doses. Mean ± SEM of three experiments. The reference line in grey shows a typical response of cells from a CS patient. **(E)** Defective RNA synthesis recovery is complemented by lentiviral transduction of primary patient fibroblasts with wild-type PCNA. RRS was measured using EdU incorporation and automated high content microscopy in cells expressing high levels of ectopic PCNA. Mean ± SEM of four experiments.

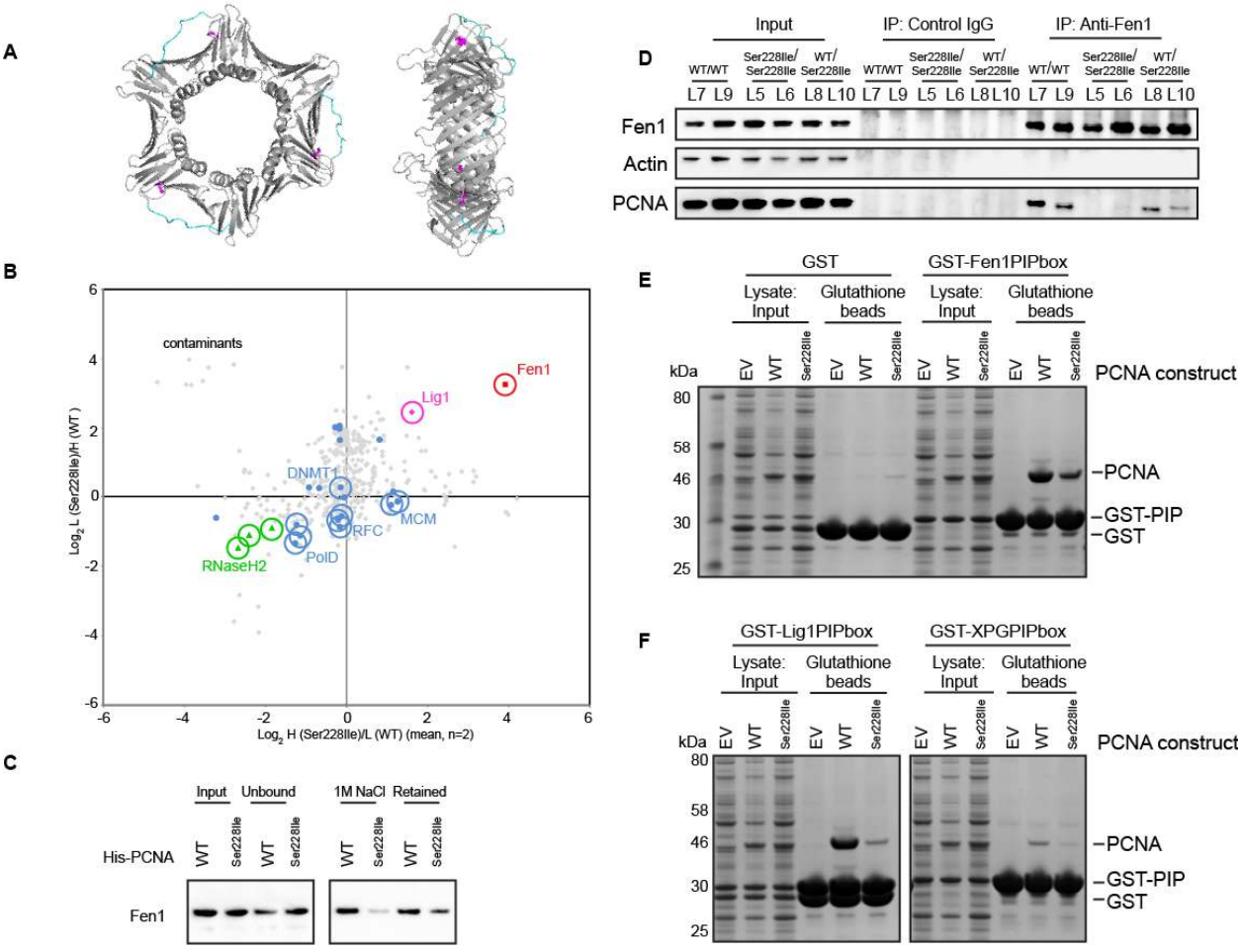

Figure 4: Perturbed PCNA interactions resulting from the p.Ser228Ile mutation. (A) Front and side views of the PCNA homotrimer, with Ser228 highlighted in magenta and the IDCL highlighted in cyan. Generated using POLYVIEW-3D (40), based on the 1YM crystal structure, rendered using PyMol. **(B)** Graphical representation of SILAC-based comparative PCNA-interaction analyses. Lysine [$^{13}C_6/^{15}N_2$] and arginine [$^{13}C_6/^{15}N_4$] labeled (H), or unlabeled (L), cell extracts were purified on wild type (WT) PCNA or p.Ser228Ile PCNA affinity columns and eluted proteins analyzed by mass spectrometry. Each point represents the observed H:L ratios of a protein present

in 3/3 experiments. In blue are known PCNA interacting proteins (Table S1). Proteins of particular interest are labeled by name. **(C)** Recombinant HisPCNA (WT or p.Ser228Ile) was added to HeLa cell extracts and proteins bound to nickel-NTA. Proteins eluted at 1M NaCl or retained on the beads were analyzed by western blot for Fen1. **(D)** . Anti-Fen1 immunoprecipitates, from extracts made from lymphoblastoid lines derived from affected individuals or family members, were analyzed by western blot for the presence of PCNA, Fen1, and actin. PCNA is almost undetectable in the precipitation from cell extracts derived from affected individuals. **(E)** *E. coli* lysates expressing empty vector (EV) or PCNA (WT or p.Ser228Ile) were mixed with lysates expressing GST or GST fused to the PIP box of Fen1. Inputs and glutathione-purified proteins were analyzed by SDS-PAGE and Coomassie staining. **(F)** A similar experiment to **E**, except GST fusions comprising the PIP box of Lig1 and XPG were used.

Table 1: A comparison of the clinical findings of individuals homozygous for *PCNA*c.683G>T and features of AT, CS and the neurological form of XP.

	VI:7	VI:9	VI:11	VI:6	ATAXIA TELANGIECTASIA	COCKAYNE SYNDROME	XERODERMA PIGMENTOSUM (neurological form)[a]
GENDER	F	F	M	F			
AGE (YRS)	11.5	14.3	26	31			
GROWTH							
Short stature	- 3.8 SDS	- 5.2 SDS	- 4.3 SDS	significant*	+	++	+
Head circumference	- 3.2 SDS	- 2.8 SDS	0.1 SDS	- 2.8 SDS	-	++	+
EYES							
Ocular telangiectasia	✓	✓	✓	-	++	-	-
Photophobia	✓	✓	✓	✓	-	+	++
Other eye abnormalities	-	-	-	UV induced conjunctivitis	Oculomotor apraxia	Cataracts, Pigmentary retinopathy, Optic atrophy	(limited to sun exposed structures) UV induced conjunctivitis, Inflammatory lesions
SKIN							
Cutaneous telangiectasia	✓	✓	✓	✓	++	-	-
Photosensitivity	✓	✓	✓	✓	-/+	++	++
Predisposition to sunlight induced skin cancer	N/K	N/K	+	N/K	-/+	-	++
NEUROLOGY							
Developmental delay	✓	✓	✓	✓	-/+	++	++
Ataxia/gait instability	✓	✓	✓	✓	++	++	+
Neurodegeneration	✓	✓	✓	✓	++	++	++
Cerebellar atrophy	N/K	✓	N/K	N/K	++	++ Also hypomyelination, Putaminal calcifications	++
Hearing loss	✓ Prelingual	✓ Prelingual	✓ Prelingual	✓§ Prelingual	-	++ Progressive, postlingual	++ Progressive, postlingual
ADDITIONAL FEATURES							
Premature aging	N/K	N/K	-	✓	+	++	++
Other physical findings	-	-	Absent pubertal growth spurt	Diaphragmatic hernia, Absent pubertal growth spurt	Increased risk of leukemia and lymphoma	Incomplete puberty, Dental caries	

Abbreviations: *, not possible to obtain accurate measurement; N/K, not known; (✓), indicates presence of a feature in an affected subject; (++), indicates traits that are hallmarks of the disease; (+), indicates traits that are sometimes associated; (-), indicates traits that are absent or not associated, [§]; aided, but not cognitive impairment prevented accurate assessment of severity, [a]; about 25% of XP patients (from groups A, D, F and G) have neurological abnormalities (the information in the table applies to the neurological form of the disorder).